6-6-2016

Design and Validate a Synthetic Circuit for Detecting Pathway Signaling in Mammalian Cells

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I HEREBY RECOMMEND THAT THE THESIS PREPARED
UNDER MY SUPERVISION BY

Darisha Jhutty, Nicholas Parker

ENTITLED

DESIGN AND VALIDATE A SYNTHETIC CIRCUIT FOR DETECTING PATHWAY SIGNALING IN MAMMALIAN CELLS

BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF

BACHELOR OF SCIENCE
IN
BIOENGINEERING

Thesis Advisor(s) Zhiwen Zhang — Date 06/05/2016

06/06/2016

Department Chair(s) — Date 06/06/2016

DESIGN AND VALIDATE A SYNTHETIC CIRCUIT FOR DETECTING PATHWAY SIGNALING IN MAMMALIAN CELLS

By

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SENIOR DESIGN PROJECT REPORT

Submitted to
the Department of Bioengineering

of

SANTA CLARA UNIVERSITY

in Partial Fulfillment of the Requirements
for the degree of
Bachelor of Science in Bioengineering

Santa Clara, California

2016

ABSTRACT

Synthetic circuits provide novel ways for scientists to program and probe mammalian cell behavior. This allows for enhanced research tools and chemical sensors. Our project works with an engineered synthetic circuit and monitors the output of the circuit using green fluorescent protein(GFP) and luciferase. Expression of GFP is both quantifiable and observable over a time period in living cells. Luciferase can be detected by an assay in lysed cells to give data on overall expression of the circuit. By measuring and comparing output of this circuit over time, we can create a model to demonstrate the lack of expression, minimal expression, and maximum expression of the circuit. Our dual reporter system can be adapted to give detailed information on various promoter elements of synthetic circuits.

This dual reporter system was designed to monitor signal pathways within mammalian cells. Signal pathways are a complex and dynamic interconnected web of protein expression. Methods to monitor these pathways *en vivo* poses a challenge due to lack of non-invasive reporting systems in desired cell models. The use of a dual-reporter system with expression of GFP and firefly luciferase allows for the assessment of signal transduction with resulting data sets of overall intensity and temporality in real-time. Neither light emitting marker could show these two properties alone. To demonstrate this system and establish a scale of expression, we transformed mammalian HEK cells with a vector coding for the dual reporter. The transformed cell lines were monitored via fluorescent microscopy and luciferase assays. Our findings show successful basal level expression of synthetic circuit dual-reporter system both qualitatively and quantitatively in a way that neither reporter protein could achieve alone. Our system can successfully deliver this data type and can be an effective tool for cell signal pathway expression.

ACKNOWLEDGMENTS

We would like to give thanks to our friends and family who supported us with our ventures throughout college and our senior design project.

We would like to extend our thanks to our advisors, Dr. Zhiwen Zhang and Dr. Biao Lu, who helped motivate us and provided us with ample scientific help throughout our senior design process.

We would also like to thank Santa Clara University's Undergraduate School of Engineering for the generous grant to fund our project.

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LIST OF ABBREVIATIONS

GFP - Green Fluorescent Protein

Luc - Luciferase

DMEM - Dulbecco's Modified Eagle's Medium

PBS - Phosphate buffer saline

CMV - cytomegalovirus

mCMV - minimal cytomegalovirus

nm - nanometer

mL - milliliter

µL - microliter

NF-κB - nuclear factor kappa beta

IL-1 - interleukin 1

TNFα - tumor necrosis factor α

IL-6 - interleukin 6

GST - glutathione s-transferase

EDTA - ethylenediaminetetraacetic acid

Tris-Cl - Tris(hydroxymethyl)aminomethane chloride

NaCl - sodium chloride

1. INTRODUCTION

1.1 Background and Motivation

The use of synthetic circuits has revolutionized the field of biology and medical research. Synthetic circuits allow researchers to test for specific proteins and chemicals both *in vivo* and *in vitro*. Using synthetic circuits, areas of biology which have been hard to test for in the past become a possibility[5]. Signal pathways within mammalian cells are a complex and dynamic interconnected web of protein expression. Methods to monitor these pathways *in vivo* pose a challenge due to the lack of non-invasive reporting systems in desired cell models[11]. Utilizing the concept of synthetic circuits, our project makes it possible for data to be collected from complex cellular process, like signal pathways. Beyond monitoring of signal pathways, there is a great need for drug screening for drug discovery. As safer and more indirect ways of administering drugs to patients are developed, it is important to monitor treatment progression[11]. Our approach to synthetic circuits allows for real time monitoring of cells in addition to signal quantification in living cells. By establishing a standard and a circuit allowing researchers to put their promoter of choice into, whether it be to study signal pathways or monitor drug delivery, we can provide a robust and easy-to-use system for monitoring signal pathway activities in a laboratory setting.

1.2 Literature Review

1.2.1 Cellular Signaling

Currently, signal transduction is one of the most widely studied areas in biology. Signal transduction, or cellular signaling, involves a complex series of molecular interactions and external proteins, lipids, and ions stimulation[10]. Extracellular information is translated into an intracellular response, often through elaborate networks of interwoven intracellular "signaling cascades"[4]. The contact with other cells or the environment can stimulate many cellular reactions, such as cell growth, cell migration or even programmed cell death, called apoptosis[10]. Cells use various pathways to communicate and elicit changes in transcription and respond to external stimuli. However, many of the pathways are not very well researched today due to the lack of appropriate monitoring tools.

In particular, inflammation is an important biological response to a tissue of injury often caused by the invasion of pathogens. The inflammatory response is characterized by activation of various signaling pathways such as NF-κB that regulate expression of both pro- and anti-inflammatory mediators in resident tissue cells and leukocytes recruited from the blood. NF-κB regulates the acute phase response of inflammation and has been considered a proinflammatory signaling pathway through activation by proinflammatory cytokines such as interleukin 1 (IL-1) and tumor necrosis factor α (TNFα), and IL-6[2]. Just as the inflammatory response is an important cell signaling pathway observed, nearly every disease or human condition also involves complex networks of other cellular signals. The proteins involved in these signal pathways are important drug targets. Therefore, manipulation and analysis of those signaling pathways and their interactions within the system and communication with other signaling pathways remain challenging.

1.2.2 Synthetic Circuits

Our project is made possible through the concept of synthetic biological circuits. The concept of synthetic circuits was developed in the early 2000's and involves natural cellular processes like gene transcription are combined in several vectors to form a 'circuit.'[1] Natural cellular elements developed by evolution over time, and despite the enormous complexity of the entire organism, most cellular processes can be broken down to interactions between common elements[7]. Synthetic circuits attempts to take those biological elements such as promoters, reporters, and vectors and create something understandable and applicable. Natural biological processes use these common elements to accomplish thousands of tasks and between all known genes for even more tasks can be found. This gives bioengineers a world of possibilities of elements to choose from.

These biological elements can be designed to function just like electrical circuits, with an input, a transduce that signal, and deliver an output.[11] By replacing the components in a synthetic circuit with several biological components, one can control the functionality of the vector. For example, the switch in the circuit can be a signaling pathway promoter, which binds a transcription factor and starts the transcription of the gene. This promoter can be triggered by specific signals, and this is where we gain the ability to study different molecules. Transcription

is the process of a cell reading a gene on a DNA strand and is required to make proteins. Then the signal is carried through the transcription of a reporter gene, similar to how a wire would, and it results in the output, which is the protein product. Through such a reporter system, synthetic circuits can be used for various applications such as gene expression, pathway signaling and more cell-to-cell communications.[7]

1.3 Current methods

Traditionally, signal transduction events have been studied by using biochemical analysis through assays such as monitoring the expression of GST-tagged proteins or other activity assays and western blotting analysis that are able to study and quantify the binding or activity of a specific biological molecule.[8] These immune-based analysis and techniques of protein phosphorylation have been a mainstay of signaling pathway analysis. Today, high-throughput assays have emerged as alternative approach for monitoring cell signaling pathways. To monitor pathway activation, fluorescent reporters are commonly used. Although these fluorescent markers allow real-time monitoring of individual responses, quantification is complicated and time-consuming because it requires specialized fluorescent cell-sorters, as they are more time-consuming and costly.[9]

To overcome this drawback, bioluminescent proteins can be used in place of GFP. In particular, the firefly luciferase-based reporter system is used as an even simpler method of quantification and may be applied to high-throughput systems.[2] Disadvantages of luciferase include the lack of visualization capabilities that fluorescent markers provide and the inability to monitor cell signaling within individual cells. Another technique is an expensive method called mass spectrometry which is able to sort molecules based on their ionic charge. However, these strategies lack specificity and sensitivity as they either do not identify the specific proteins involved in the signal pathways or do not produce highly amplified signals.[2] These studies require multiple complex steps and also must be conducted in chemical solutions, or in-vitro, and do not fully represent the true human condition. In addition, many of the current experimental procedures for viewing signal pathways over time document signal transduction snapshots of individual events only without the benefit of visualizing the whole stage simultaneously.[8]

1.4 Project Highlights

1.4.1 Project Goal

We will provide a novel solution to study the signal transduction events in cells. To combat the disadvantage of current solutions, cell behavior assays need to be developed that can be quick, easy, and used on living tissues. It must also be able to be quantified to measure exact expression levels. Our goal is to utilize a synthetic circuit for real-time monitoring and quantification of pathway signaling. Our approach will allow for a more robust, frugal, quick, and easy method to monitor signal transduction in human cells.

1.4.2 Design Proposal

Our project proposes a dual-reporter system inserted into a genetic circuit that expresses two different reporter chemicals. Green Fluorescent Protein (GFP) is a commonly used fluorescent marker. Although it can be easily tracked via a fluorescent microscope, GFP is most effective at mapping the expression qualitatively.[9] Fluorescent molecules like GFP suffer from an autofluorescent effect and can obscured by other fluorescent molecules in a cell. Luciferase, an oxidative enzyme used for bioluminescence, will be used as the second reporter for the quantitative analysis. Luciferase is more difficult to visualize in living cells due to the oxidative reagents being necessary for its light emission.[2] The use of a dual-reporter system with expression of green fluorescent protein and firefly luciferase allows for the assessment of signal transduction with resulting data sets of overall intensity and temporality in real-time. Neither luminescent marker could show these two properties alone and each makes up for the shortcomings of the other.

To gauge the dual-reporter system performance, we will perform a series of tests of how effectively the genetic circuit have infected the cell and activated the visual responses. These visual cues will be detected by fluorescent microscopy and the use of a plate reader to produce qualitative and quantitative signal expression data. The use of these instruments will allow the measurement of both the expression of GFP in the cells and the expression of luciferase after the signaling pathway is activated. If GFP and luciferase expression provide meaningful qualitative and quantitative data in living, human cells, our system will be validated. The use of vectors which contain differing levels of signal expression will be used to test the signal strength and

sensitivity of our system to determine if it is more effective than conventional methods. Additionally, GFP quantification will be compared to luciferase quantification to validate that both reporters are necessary and provide superior data to a single-reporter system. With this, the discussion and further steps will be linked with the results obtained from these research tests and studies.

1.4.3 Expected Results

To validate our dual-reporter synthetic circuit as an effective tool for studying signal pathways, it must meet a variety of conditions. Our system must be active in living, human, cells and continue to work overtime as expression changes. It must be able to provide visual feedback to researchers on the change in expression level in real-time. We expect to see these results in the form of fluorescence changes seen in cells over time when visualized using a fluorescent microscope. This is can show our system's ability to show pathway activation in response to stimuli. Our system must also be able to output a signal which can be quantitatively measured by researchers in a lab setting. We expect that when analyzed using biochemical quantification assays, our system will give significant data upon its expression relating to the magnitude of the expression.

The validation of our system involves the use of vectors which have different levels of signal promotion. We expect that cells with no promoter will express no signal, and cells with a minimal promoter in their circuit will show less expression than cells with an enhanced promoter. It is also expected that they will show greater fluorescent and luminescent signals than cells without our system. Both the visualization and assays of our cells should show a similar trend in expression level between our vectors. To validate the use of luciferase for quantification over GFP, we also expect to see a greater signal and greater sensitivity in the luciferase assay over the GFP assay. With the expression measured as we expect, we hope to validate our system as a novel system which can be used to detect signal pathway expression.

1.5 A back-up plan

The validation of our system could vary in degree of completeness. Our goal is to show its ability to output a signal in a superior way to existing methods which meets our self-defined parameters, but the extent to which we demonstrate this expression could be reduced or adjusted if our expected results are not seen. If we are not able to validated every aspect of our project, for example, if we cannot show that our system is able to determine the difference between minimal and promoterless expression levels, we can state the extent of our sensitivity. In that case, using vectors with different expression levels could help fill in the gap about just how sensitive our system is.

Another area which requires a backup plan is in the validation of the use of two reporters. Based on our literature search, we assumed luciferase would produce a clearer signal to GFP when quantified. Although we hope to prove the increase in quantification ability of luciferase over GFP, if we find this is not the case, and GFP shows expression levels more precisely, we will have to consider the use of a single-reporter system. Single-reporter GFP systems commonly used, and so this will make our system less novel, but it is a scenario we are prepared for. If we find after multiple quantification assays that GFP is superior to luciferase in both visualization and quantification, we will move forward by showing just to what extent it has an improved ability and why, and the work to design a single-reporter system and validate it.

1.6 Significance

We will provide an innovative solution to study the signal transduction events in cells. To combat the disadvantage of current solutions, cell behavior assays need to be developed that can be quick, easy, and used on living tissues. Our goal is to utilize a synthetic circuit for real-time monitoring and quantification of pathway signaling. Our approach will allow for a more robust, frugal, quick, and easy method to monitor signal transduction in human cells. Our product will be useful for expanded research in synthetic biology and cell-pathway signaling with substitution of different promoters within our genetic circuit. Researchers will be able to use our system to monitor complex cellular responses in real time and gain new insights in how human cells behave on an intracellular level. Furthermore, our system can be used in academia at medical

schools, graduate schools, and educational biology labs. Although we designed our system to help study signal pathways, the concept of dual signalling itself is a very useful system and can be used as a reporter for other areas of research such a drug screening, cancer research, or any area that uses real-time cell sensing.

1.7 Team Management

Senior Design Team:

Darisha Jhutty - Biomolecular Track, Bioengineering

Nicholas Parker - Biomolecular Track, Bioengineering

Advisors:

Dr. Zhiwen Zhang - Associate Professor, Bioengineering

Dr. Biao Lu - Assistant Professor, Bioengineering

1.8 Budget

We received $1000 in funding for this project provided by the Santa Clara University School of Engineering. Additionally, we made extensive use of Dr. Lu's and the tissue engineering lab and its associated equipment and supplies. See **Appendix B** for a budget table.

1.9 Project Timeline

In the fall we were trained to learn the proper laboratory techniques. This involved refining protocols in a tissue lab for use with HEK293 cells. In the winter we ran our experiments and collected visualization data and quantification data from luciferase quantification time course. In this time we also began searching for buffers to help us quantify GFP for our validation of the use of two reporters. Over the course of spring quarter we ran our comparative time course validating our use of two reporters and analyzed our results, creating graphs and compiling our data. We also prepared for the senior design conference in May and continued to work on our thesis up until June. A rough estimate of our workflow can be found in **Appendix A**.

2. SYNTHETIC CIRCUIT DESIGN

2.1 Introduction

As mentioned earlier, synthetic biological circuits truly provide structure to our dual-reporter system and study cellular signaling. Our dual reporter system will be expressed in response to transcription factors which are involved in the signaling pathway. The transcription factors will activate a promoter region in the sequence of DNA we are inserting into the mammalian cells. The operon region will contain multiple copies of the promoter to increase gene expression. This is essential as transcription factors leading to transcription are the ultimate product of cellular signaling. Cellular signaling is the preliminary step that leads to signal transduction inside the cell, activation of the transcription factors, and lastly binding of the transcription factors to the transcription factor elements on our circuit.[8] The result of this design leads to the high expression levels of the end products, GFP and Luciferase, which allows for real-time monitoring of the signal and quantification of the signal.

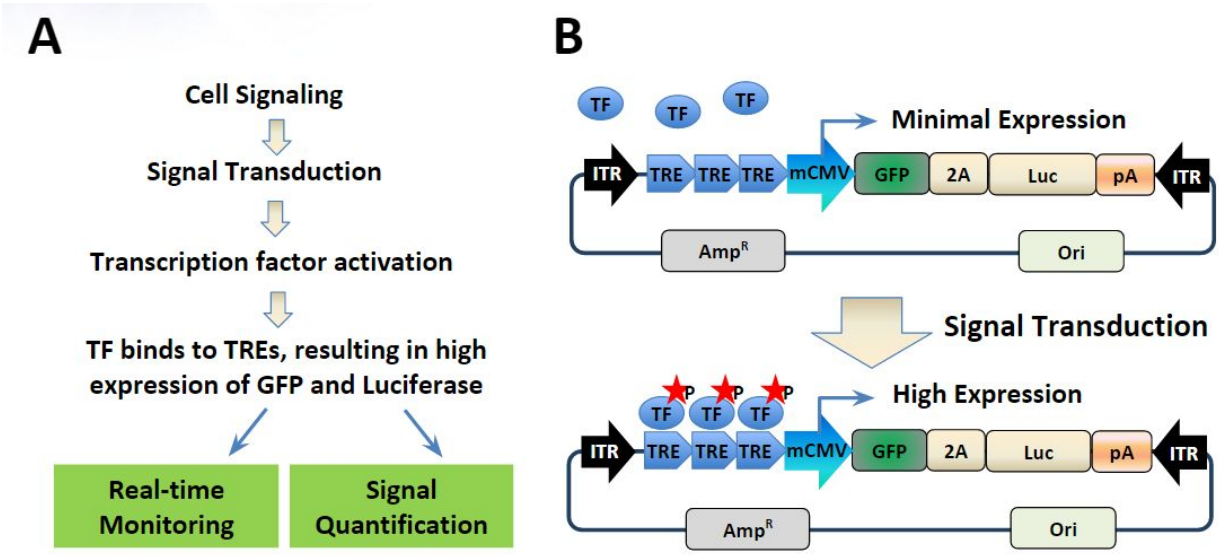

Figure 2.1. Overview of system design of dual-based reporters. (A) General flow of cellular signal pathway transduction. After transcription factors are expressed dual-reporter sequence (shown 1B) expresses GFP and luciferase for both real-time monitoring and signal quantification. (B) Schematic representation of the main features of the dual reporter system. The dual reporters, GFP and firefly luciferase (Luc) separated by a self cleavage peptide (2A) under the control of a minimal expression promoter (mCMV). The insertion of transcription factor response elements (TFs) are done through multiple cloning sites. TFs are activated through stimulation (*P) and bind to transcription response elements (TREs) resulting in high levels of expression.

2.2 Design Specifications

Seen in **Figure 2.1 (B)**, diagram of the vector setup we used for our synthetic circuit. What these loops represent are a circular piece of DNA, called a vector, which we put into our cells to express our GFP+Luc gene. The switch/promoter for our circuit is activated when transcription factors bind, and cause the expression of the gene output of GFP+Luc to be expressed and visualized. Transcription factors are the molecules we are testing for. They bind to our promoter and flip the switch to activate our circuit. The other elements are needed for it to coexist with the cells and create function proteins. What will happen when our product is used to test a particular molecule (which will be acting as the transcription factor), when the TFs bind, like in the lower picture, we will see fluorescence and luminescence, this is scenario is shown on the bottom, and when the correct transcription factors do not bind, we will not see anything.

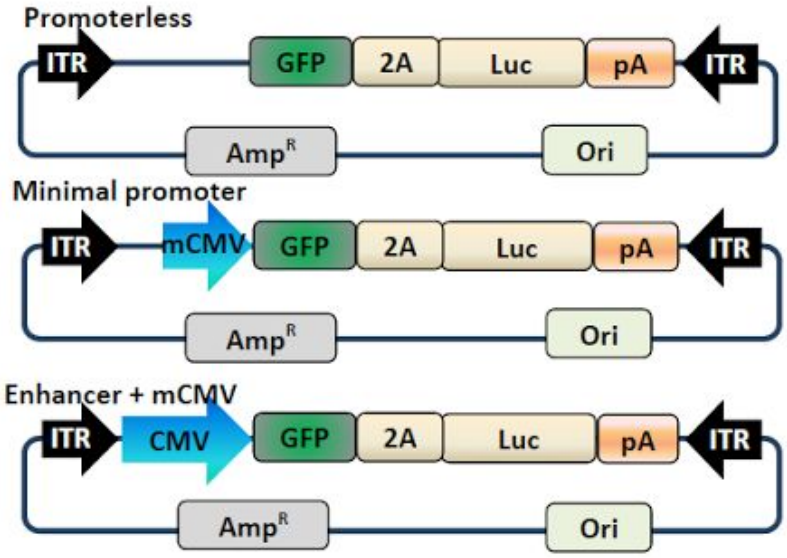

Figure 2.2. Plasmid design. Design of our plasmids with GFP and Luc genes. A promoterless, minimal promoter, and enhanced promoter allows us to gauge various expression levels.

In **Figure 2.2**, the 3 expression vectors of the synthetic circuit are depicted. All 3 vectors have both reporters GFP+Luc following the respective promoters. In addition to the GFP and Luc genes, a peptide fragment (2A), poly-A signal, and ampicillin resistance gene was inserted

to increase efficiency of translation and assist in selection capabilities. But to develop a baseline and determine sensitivity in our system (detect the difference between high expression and low expression), we used three different promoters that are permanently 'switched on' or constitutively expressed. One vector is composed of no promoter in order to establish a control and determine any noise/background to our regular synthetic circuit. The next vector had a minimal promoter, mCMV, which expressed small amounts of both the reporter proteins to see if we could detect it at a low expression level. The last vector contained an enhanced promoter which would determine high expression levels when our system is saturated with analyte. Since our vectors are all constitutively expressed, each protein, GFP and Luciferase, were expressed at equal levels as their respective promoters to study ideal conditions. In terms of application, these three vectors allow us to compare our results in respect to other systems of varied expression levels.

2.3 Materials and Methods

We conducted several experiments to validate the design of our circuit. The 5 main goals for these validation steps were to ensure we are able to perform live cell monitoring, able to quantify the respective gene product (in our case, GFP and luciferase), detect any noise and background to our received signal, test the repeatability or robustness of the system, and measure the comparative sensitivity of each of the vectors (promoterless, minimal promoter, and enhanced promoter) to generate a basal level of expression.

To demonstrate this system and establish a scale of expression, we used mammalian human embryonic kidney cells to appropriately test signal pathways as they would be in humans. To culture our cells we used common cell culturing supplies. We used transfection using a chemical reagent, a common biological procedure, to insert the with our 3 circuit vectors coding for different signals of the dual reporter in response. The transfected cell lines were monitored via fluorescent microscopy and biochemical assays.

3. VISUALIZATION

3.1 Introduction

Green Fluorescent Protein has been studied in detail and widely used in molecular biology and bioengineering and comes from a gene taken from a species of deep-sea fluorescent jellyfish. GFP is used as one of the reporter genes in this dual reporter system, as seen in our synthetic circuit (DNA vector) of an organism so that specific cells will express light under certain conditions. The fluorescent ability of this protein means that when light of a certain wavelength is shined onto the protein, it will emit green light back, which can be visualized. This tool can specifically be used as biomarkers or to tag certain genes or cells. Using this method, exact proteins or transcription factors and cell signalling pathways involved can be traced and studied.

3.2 Design Requirements and Constraints

For the visualization experiments, live transfected HEK293 cells GFP expression was monitored via microscopy in 6-well plates using a LEICA fluorescent microscope. Data was processed using LAS 3.8 software where images of both control and fluorescent images of the cells were captured. A possible drawback with the use of GFP is that it requires an excitation light source to emit its light, and in cells with GFP, often other molecules will react to the same light and obscure the data.

3.3 Results and Discussion

Several control experiments were repeated in order to compare relative levels of cell density for each vector to relative fluorescence **(Figure 3.1).** From these experiments, we determined that the enhanced promoter was showing the greatest amount of fluorescence, and subsequently each vector showed a linear decrease in expression levels. As seen in the control, there was no fluorescence detected, meaning that the background/noise levels are low. Thus, we can conclude that the vectors were properly functioning, and GFP was expressed in the cells at different levels. Refer to **Appendix H** for more relevant GFP control and fluorescent images.

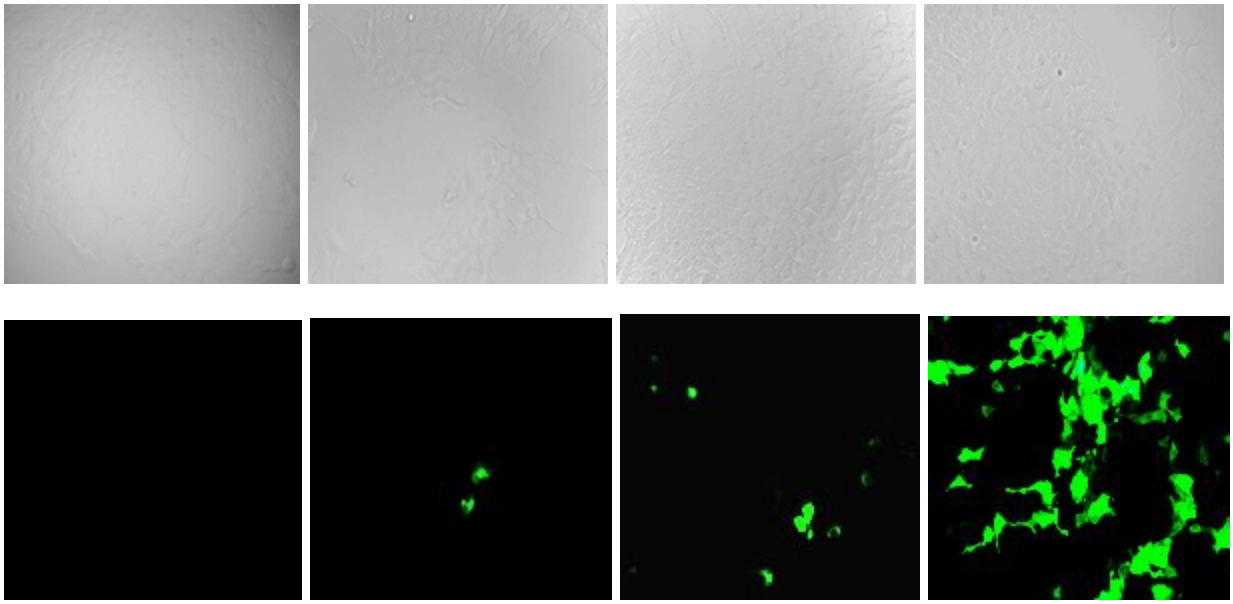

Figure 3.1. Fluorescent images versus the control at T=48 Hours. From left to right, cells transfected with the promoterless plasmid, minimal promoter, and enhanced promoter.

After determining that GFP was being expressed at different levels for each vector type, we conducting a time course study of GFP expression over 72 hours at 3 separate time points **(Figure 3.2)**. As time passed by, GFP concentrations increased as can be seen in the 72 hour versus 24 hour time points. In addition, the previous GFP visualization study was further validated as the enhanced promoter after 72 hours demonstrated a higher GFP signal than the minimal promoter and promoterless. We conclude that GFP is a successful tool over time measurements and in living cells.

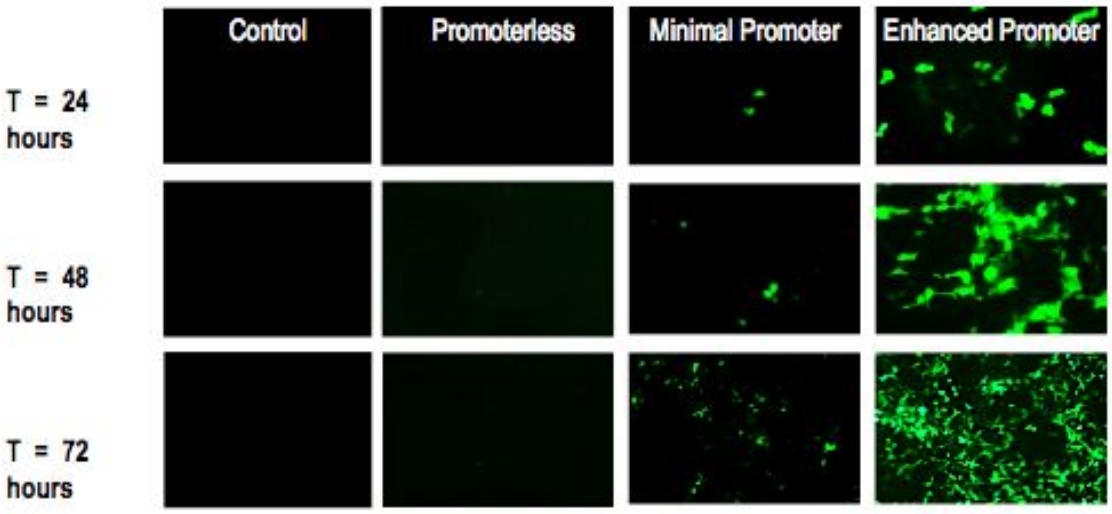

Figure 3.2. Fluorescent images versus the control at T=24, 48, and 72 hours. From left to right, cells transfected with the promoterless plasmid, minimal promoter, and enhanced promoter.

4. QUANTIFICATION

4.1 Introduction

Beyond the ability to be visualized, the dual-reporter system will be able to be quantified to give exact information on the expression of the synthetic circuit. Visualization of GFP in living cells is a powerful tool to help researchers determine when the circuit is being expressed, but that information needs to be recorded in exact quantities to be compared. While the experiments with the various vectors showed a clearly defined difference in the GFP expression between cells transfected with the promoterless vector, minimal promoter, and enhanced promoter as well as the control, we cannot be certain as to the exact amount this expression differs using the fluorescent images in Figure 3.2. To show the quantification ability of the system we attempted to quantify both GFP and luciferase to see if it showed the same trend we qualitatively saw from the visualization experiments and then ran both side-by-side to make sure the conditions were correct.

Luciferase is a chemiluminescent molecule taken from fireflies (*photinus pyrallis*) which reacts in the presence of O_2 and ATP to produce light.[2] The mechanism works by a two-step process in which luciferin becomes luciferyl adenylate and then oxyluciferin. When the ATP

ratio in a cell is high, ATP binds to a carboxylic acid on luciferin making luciferyl adenylate.[2] The addition of O_2 then creates an energetically excited state oxyluciferin which releases light as it goes back to a stable, ground state[7]. Unlike GFP, other molecules in a cell are not excited by the same addition of ATP and O_2, so there is less background light which could obscure the signal. This is why luciferase was specifically used in the system for quantification. Additionally, it was important for us to attempt to quantify both molecules to validate the system's need for both GFP and luciferase. If GFP was readily quantifiable in addition to its ability to be visualized using a fluorescent microscope, then it would be unnecessary to have the dual-reporter system. Thus, we conducted validation studies to prove the need for a dual-reporter system with GFP and luciferase over a single-reporter system of just GFP.

4.2 Design Requirements

4.2.1 Luciferase Assay

Luciferase was quantified using the Promega Luciferase Assay system. The Promega Luciferase Assay System includes Passive Lysis buffer to lyse the cells and Luciferase Assay Reagent which provides the chemical environment needed to promote luciferase activity. This reagent includes the ATP and O_2 need to complete the reaction and allow the luciferase to become oxyluciferin and emit light. The lysate with the substrate is loaded into the Tecan Infinite m200 plate reader and the light expression is recorded using the "Luminescence" setting. Luciferase assays are easy to obtain for most research labs, and plate readers are also commonplace in biology labs. It is important that the reporter molecules were accessible to researchers since they are the target user for this system. The luciferase assay kit was tested by performing preliminary quantification tests on the HEK293 cells with the three synthetic circuit vectors. This preliminary test allowed us to see that the kit itself worked and we were familiar with the protocol.

4.2.2 Quantification Time Course

After the preliminary experiment, we ran a time course which involved visualizing GFP in all four cell types and quantifying luciferase in the same cells. The time course data was taken at 24, 48, and 72 hours. The quantification experiments were conducted by running a time course and checking quantification levels in 24 hour intervals. HEK293 cells were grown in the same

conditions and separate plates were transfected with the three different vectors and a portion of the wells were not transfected to be a control. 24 hours after transfection, a well of each cell type (control, promoterless, minimal promoter, and enhanced promoter) were visualized under a fluorescent microscope to detect GFP. Afterwards, these cells were assayed to detect quantities of GFP and luciferase. Both visualization and quantification were conducted on the same cells to ensure that the side-by-side comparison could be seen. A more detailed explanation of the time course can be found in in the appendices Experiment 3.

4.2.3 GFP Quantification Challenges

Initially, we attempted to quantify GFP in a number of ways. Living cells which had been transfected with the vectors and were expressing GFP were loaded into a plate reader and a scan was taken using the Tecan plate reader's "Fluorescence" setting with the GFP excitation frequency. The data from this experiment failed to show any qualitative trend with the GFP expression we could see using the fluorescent microscope. To match protocol of the luciferase assay quantification experiments, we tried to quantify GFP using the lysate from the same Passive Lysis Buffer. Lysate from cells expressing the system was loaded into a black 96-well clear bottom plate and the plate reader excited the samples and read the emission. This method also failed to produce any trend in the GFP quantification data.

Assuming that the GFP protein was inactivated by passive lysis buffer or that the signal was somehow weakened by the protocol in appendix F, we then tested a series of lysis buffers found via a literature search to see if they could lyse the cells and allow GFP to stay active and be read accurately by the plate reader. A lysis buffer of Tris-Cl with 150mM NaCl, 0.5mM EDTA, and .5% Tween 20 at a pH of 7.5 was used as well as the same buffer with PMSF added. PMSF, or phenylmethane sulfonyl fluoride acts as a protease inhibitor and stops the cell's natural protease activity which occurs upon lysis. We hoped that PMSF would inactivate the cellular proteases and allow GFP to stay active after lysis. HCl was also tested as a possible lysis buffer. More details on the buffers tested can be found in appendices Experiment 4.

The various lysis buffers showed no trend and so a GFP Quantification Kit was purchased to quantify the protein reporter expression levels. The GFP Quantification Kit was purchased from BioVision and contained a GFP Assay Buffer to show GFP activity. It was set

up in a way very similar to the luciferase assay buffer. Lysate and the specific assay buffer were loaded it into a clear bottom black 96-well plate to be measured by the plate reader. The lysate was read by Tecan plate reader and relative fluorescence levels were taken. After a preliminary experiment to test the effectiveness of the GFP quantification kit, the GFP quantification was run side-by-side with the luciferase quantification. A time course was set up using control cells and cells transfected with the promoterless, minimal, and enhanced promoter vectors. Twice as many wells of culture were used as the last experiment to allow for cells to be used for both GFP and luciferase quantification. This time course was assayed to determine the amount of luciferase and GFP in the system every 24 hours after transfection for 72 hours total. A more exact explanation of this experiment's protocol can be found in appendices under Experiment 5. By using cells grown in the same conditions and looking at the quantification of both GFP and luciferase side-by-side we were able to validate that the dual-reporter system could successfully quantify the signal in a superior way to a single-reporter system.

4.3 Results and Discussion

4.3.1 Luciferase Quantification

The first time course yielded engaging results which validated the quantification ability of the dual-reporter system's ability to quantify as well as visualize. As discussed in Chapter 2, the 72 hour time course provided images taken by the fluorescent microscope which showed an increase in GFP expression with each cell type as expected. The promoterless showed some expression over control, minimal showed some over promoterless, and enhanced showed some over minimal. The data taken from the luciferase assay also showed the same trend.

Time:	24	48	72
Promoterless	221	14974	16710
Minimal	2167	66038	55459
Enhanced	124228	4688056	4693709

Figure 4.1 A time course of relative fluorescence levels (Experiment 3). Promoter types are listed on the left column and the results from the triplicate time course over 3 days are shown on the right. Data is shown in Relative Fluorescence Units taken from the Tecan plate reader. Units are normalized against the control cells which were used as background.

All vectors showed a significant rise in expression over the control cells which shows that we were able to quantify the same expression which we saw in the visualization experiment. Comparing the normalized relative fluorescence data taken from the time course as shown in Figure 4.1, it can be seen that each vector type tested showed a significant increase in quantification along with the expected trend. The promoterless vector was used as a baseline, since it has no promoter and is used in these experiments as a negative control. The fact that some luciferase was found in cells with only the vector and no promoter shows that the vector may be a bit "leaky" or expressing despite not having a promoter. Cells transfected with the minimal promoter vector showed an average of a 6-fold increase in luciferase expression over cells transfected with the promoterless vector, and cells transfected with the enhanced promoter showed an average of a 385-fold increase in fluorescence over the promoterless. This data shows that the system was able to quantify the luciferase expression. By seeing the incremental increase in the promoterless, minimal, and enhanced promoter cell expression of luciferase, the sensitivity of the synthetic circuit is also validated, since it is possible to clearly see the difference in expression under all three conditions.

4.3.2 Luciferase compared to GFP Quantification

The second time course compared the quantification ability of luciferase to the quantification ability of GFP. Luciferase quantification had a stronger and more sensitive signal, showing that the use of luciferase as the second reporter is needed to give the system its quantification ability. Running the time course and quantifying luciferase using the Promega Luciferase Assay Kit and the BioVision GFP Quantification Kit side-by-side under the same conditions was able to show us precisely each signaling molecule could be quantified.

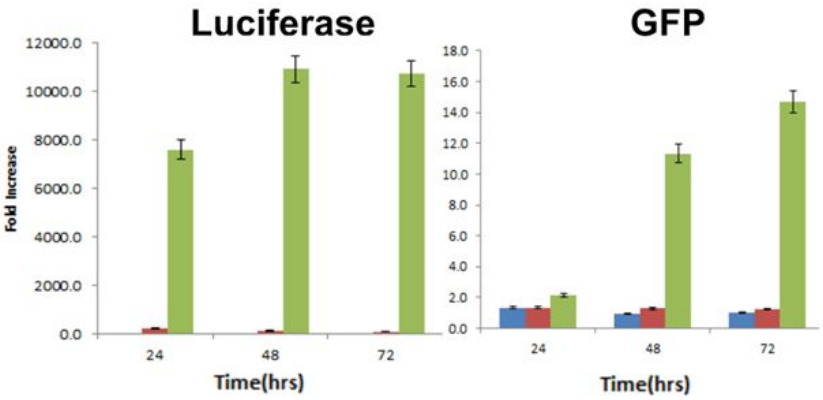

Figure 4.2 A time course study of relative fluorescence and luminescent levels (Experiment 5) Compared. The bars represent the normalized relative fluorescent or luminescent data taken from the Tecan plate reader when compared to the control. The blue bar is data taken from the cells with the Promoterless vector, the red from the minimal promoter, and the green from the enhanced promoter. The y-axis is shown in fold-increase over the control which relates to signal strength.

Figure 4.2 shows the signal strength of luciferase is greater than that of GFP. Cells with the enhanced promoter showed a 10,000-fold increase in relative luminescent units over the control. When looking at the fluorescence, only a 10-fold increase was seen comparing the same cells. The ability to differentiate a positive signal from a negative control is important, and the ability for luciferase to show that over allows for more clear results when using our system. Quantifying luciferase also produces a more sensitive system, which showed a clear difference in expression levels between the system expressing at a low level, represented by the minimally promoted vector, and a system not expressing. Our data showed that there was a 150-fold increase in signal between the cells with minimally promoted vector and the cells with no vector, and a 36-fold increase in cells with the minimal promoter over cells with the promoterless vector when luciferase was tested for. This clear increase in expression at each level. When GFP was quantified, there was almost no difference between expression levels between the control, the promoterless, and the minimally promoted cells. Figure 4.2 shows how both the promoterless and the minimally promoted cells saw a fold increase of around 1, which equates to no increase. This shows that GFP cannot determine expression at low levels.

The reason why GFP was not used alone in a single-reporter system is to be able to have the superior quantification ability of luciferase. When comparing the quantification ability of the

two, luciferase is seen output both a stronger, and more sensitive signal than GFP. A reason for this could be the autofluorescent effect of GFP. When cellular lysate is excited at the excitation frequency of GFP, many of the other molecules in the lysate may excite and emit light. While GFP is known to emit this light, at low levels it may not be enough to be distinguished from the other fluorescent elements of a cell despite the difference being apparent qualitatively through the visualization experiments. This could explain why cells with the promoterless vector and the minimally promoted vector showed similar RFU levels to that of control cells without GFP gene present in them. Overall, our experiments validated the quantification ability of our system and need for luciferase as the second marker in our dual-reporter system.

5. SUMMARY AND CONCLUSION

5.1 Summary

Our synthetic circuit is composed of two reporters, GFP and Luciferase, that can be used to monitor cellular signaling. To gauge the dual-reporter system performance, we performed a series of tests of how effectively the reporters activated the visual responses and could be quantified. These visual cues were detected by fluorescent microscopy and the use of a plate reader. The use of these instruments allowed the measurement of both the expression of GFP in the cells and the expression of luciferase after the signaling pathway is activated. In our hypothesis, if GFP and Luciferase expression levels match pathway activation, the system will be validated. We measured levels of GFP and luciferase over time and quantified each to determine sensitivity. With this, the discussion and further steps will be linked with the results obtained from these research tests and studies.

5.2 Conclusion

Based on all the data we saw, our system was validated as a successful dual-reporter system for potential pathway signaling applications. GFP was successful in visualizing expression of our circuit. It was able to monitor real-time expression of the signal in living cells under a fluorescent microscope. When luciferase was used in an assay, it was able to quantify, just how much that system was expressed, giving researchers another data point. Furthermore, we validated our system by comparing the quantifiability of GFP to Luciferase to confirm that

both are needed to give these two unique data points. Our quantitative data from luciferase expression allowed us to make a curve of relative expression that can be compared to to determine to what level the circuit is being expressed. When used to monitor pathways, the system will be able to be compared to the data we got from promoterless, minimally expressed, and enhanced promoter expression to determine to what level the pathway is being expressed. Our system has been validated and can be an incredible helpful product to researcher giving them a new way to monitor various cellular systems.

5.3 Future Work

With the completion of this circuit containing the dual-reporter output, we could create a do-it-yourself kit which would broaden the scope of the system by allowing others to add these reporters to various other promoters. Because it is so effective at both tracking expression overtime and quantitating that signal, the plasmid would additionally have various uses for biology labs and biotech companies. The eventual kit would contain our circuit in the form of the plasmid with the dual-reporter elements as well as a scale of the relevance of expression using our data from this project. A flow-chart of the kit's potential usage is shown below.

If GFP and luciferase expression levels are close to our minimal promoter samples, researchers will be able to state that there is low expression, whereas if they resemble the numbers from our enhanced promoter samples it will show that that pathway of interest is expressing at a very high rate. Although we are in the discovery stages and do not plan to go the commercialized level and create this kit, the possibilities and boundaries of this project are expansive. Our system has been validated and can be an incredible helpful product to researcher giving them a new way to monitor various cellular systems.

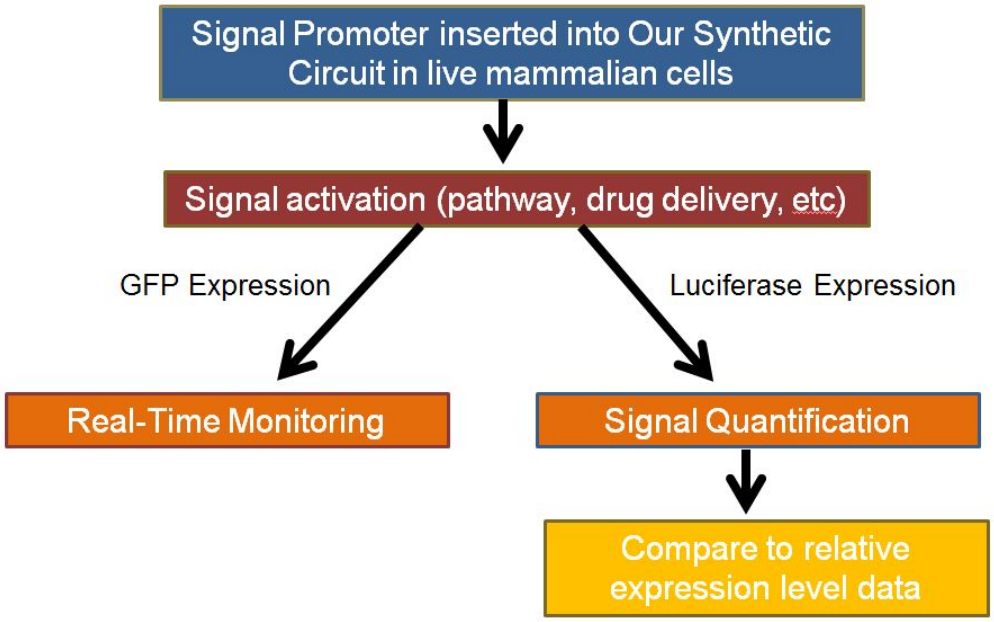

Figure 5.1 Flowchart of dual-reporter kit's usage to researchers. Starting at the top, this shows how researchers can use the information from our project and the components of the kit we hope to develop to monitor and quantify their samples. The last step on the bottom right shows how they will use the data from our project to find the relevance of their data by comparing it to our expression levels.

5.4 Potential Applications

Taken forward, our system can be used to improve measurement of a wide array of biological systems. Although our system was originally designed for detecting signaling pathways, our system can be applied to even more complex and not-understood elements in living cells to help us understand how our cells respond to various situations. Because this system can be performed in living cells, it can provide relevant in vivo data which other systems may not be able to provide. For example, we can use this system is its ability to monitor drug screening. By using a promoter for the system which activates when drugs are present, our system can be studied to quantify the amount of drug delivered in living cells. Currently, a major challenge of cancer therapy is its ability to selectively deliver drugs to specific, diseased areas. Our system would work especially well for this purpose, and be able to provide cancer researcher relevant data about if the drug was delivered in real time to the correct cells and not to any unintended targets. Gene expression can also be monitored as well, since synthetic circuits are based off the concept of gene expression. Synthetic circuits can effectively be used to test for

relevant human genetic expression such as gene response to environmental factors or genetic diseases.

6. ENGINEERING STANDARDS AND REALISTIC CONSTRAINTS

6.1 Project Justification

We believe the research and development of the dual-reporter system applies to the Santa Clara Engineering School's vision by specifically expanding upon the scientific community and advancing the current state of technology. By combining our Jesuit education and practicing our technical skills for this project, we will be prepared for professional excellence, and also provide a product that may provide service to community as a whole. Along with the SCU strategic vision, our project attempts to use scholarship to in the Jesuit tradition to help people through the medical applications of studying inflammation pathways and possibly expanding into further biological systems. This system can be adapted not only for greater understand of the human body, but also as a means to track disease pathways like cancer, monitor pathogens in water sources, or other biological study. Our project pulls from ideas of the scientific community as a whole and our system will hopefully give back as we can publish our findings in journals for medical professionals and bioengineers to draw upon. In the end, this project will help us pursue a passion for lifelong learning and continue Santa Clara's mission to be leaders of competence, conscience, and compassion.

6.2 Social Impact

Our project will allow researchers to study pathways in a new ways and this will benefit medical research. In specific, the use and of embryonic cells can be seen as a concern in the general public with those of different cultures, religions, and beliefs. After weighing the benefits of human embryonic kidney cells in comparison to other cell types, we decided that the overall social impact of this tool would help the view of embryonic cell lines. With more tools to explore intracellular and intercellular communication, it can lead to a greater understanding which can aid in first understanding, and then treating disease. Hopefully, our system can be used to help

scientists expand the horizons of medical research, and eventually assist with treating disease and helping people.

6.3 Health and Safety Concerns

Our system has the benefit of being useful only in a biological research setting. By responding to cell signals, it can only be used to measure effects on a cell. This, ethically, allows our system to only further researcher's understanding and be of benefit to society. As far as the research that went into developing this system, we adhered to strict safety regulations and disposed of all hazardous materials in the proper locations so as to minimize damage to others and the environment. The HEK cells divide without need for a donor, so there was no detriment to anyone to obtain or culture them. Proper sterile technique was used so as to not spread any transfected or other human cells and allow them to come into contact with individuals.

6.4 Economics and Manufacturability

In the current form, our system is not marketable. Our product will be useful for research within cell pathway studies, and which, furthermore, can be used in academia at medical schools, graduate schools, and educational biology labs. The dual signalling pathway itself is a very useful system. With the completion of this plasmid containing the dual-reporter system, we could create a do-it-yourself kit which could broaden the scope of the system by allowing others to add these reporters to various other promoters. Because it is so effective at both tracking expression overtime and quantifiably, the plasmid would additionally have various uses for biology labs and biotech companies. The eventual kit could contain the plasmid as well as a scale of how much expressions relates to what quantity of expression.

Although we are in the discovery stages and do not plan to go the commercialized level and create this kit, the possibilities and boundaries of this project are expansive. The results of our project have validated that it functions as planned and provides important visualization and real-time data. That validation is important to understanding the technology. Our next step with the kit is what would make our system relevant to a market. Like many biotech products such as assay kits, the real science is only part of the kit, but it can be marked up significantly. Our kit

would include the vector with our circuit and our constitutive expression information, but besides that the other components would include buffers and enzymes used to insert the desired primer. The markup on these elements could be huge and profitable depending on how commonplace the dual-reporter technology gets. Production of the kit would start at a single-lab level with scientists measuring out the buffers and components and isolating the vector. If profitable, the operation would expand to larger scale and high-throughput or assembly line.

6.5 Overall Ethical Considerations

Overall, we took the possible concerns that could arise downstream from our project and considered all potential cause and effects. Even though success is eventual through utilization of our developed system in research laboratories and institutes, we believe it is important to be thorough when dealing with the ethical concerns of many. Since many people are affected by different diseases and conditions, the availability and usage of this tool can be used for different intentions, and it would morally unjust to avoid the engineering standards associated with this project.

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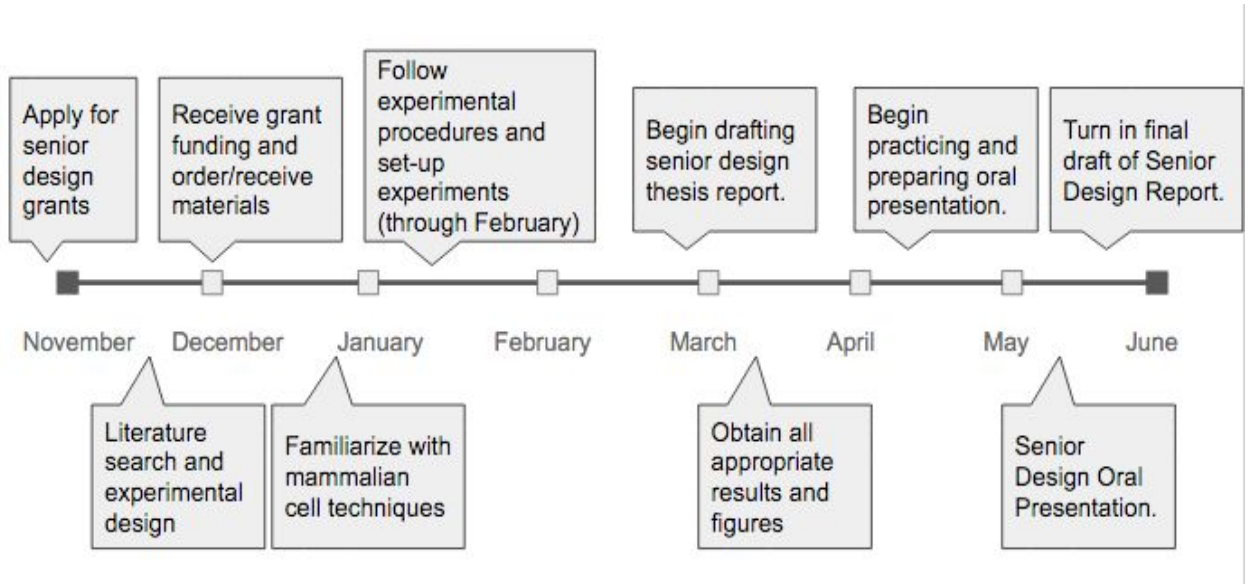
APPENDICES



A. Estimate of workflow timeline. This diagram shows when various checkpoints of our project were expected to occur. Our project followed this expected timeline with exceptions of the experiments lasting until March.

B. Budget Specifics

Supplies	Cost
Cell Culturing Supplies - Pipette Tips - Cell Media - Buffers - HEK293 cell lines - Fetal Bovine Serum	$300
Synthetic Circuit Vectors	Provided by Advisor, Dr. Lu
Imaging Supplies - Well plates - Microscope usage, plate reader useage - Luciferase assay kit - GFP Assay Kit	$700
Total	**$1000**

C. Standard Protocols

Cell Passaging

Purpose: To transfer cells to a new plate or when cell confluency is at 50-70%

1. Aspirate old media by tilting the plate towards you to prevent removal of cells. Touch tip of aspirating pipette to media.
2. Wash cells by adding 5mL of PBS to the side of the plate. Tilt plate back and forth and aspirate off the PBS.
3. Detach cells from the plate by adding 1mL of trypsin drop-wise directly onto the cells. Place in 37° C incubator for 3 min, check detachment, and then place back in if necessary.
4. Add 5mL of DMEM + 10% FBS media to plate. Tap plate to dislodge and pipetted media and cells from the bottom of tilted plate to the top to dislodge.
5. Transfer cells to 15mL centrifuge tube. Spin down at 1000rpm for 5min.
6. Aspirate media off.
7. Resuspend cells in 6mL of DMEM+10%FBS
8. Add DMEM+10%FBS to your plate according to its size
9. Transfer 1-2mL to each 6mm plate or 50 µLs to each 12-well plate well or 100 µL to each well of a 6-well plate. Tilt plate back and forth to spread cells.
10. Label plate with name, date, cell type, and passage number and put in the incubator

Cell Thawing

Purpose: To grow HEK293 cells which were previously in cyrotubes

Used HEK293 Cells purchased by Santa Clara University

1. Remove Cryotube from -80° C Freezer
2. Place in 30° C water bath incubator, until thawed to point of one crystal left (1-2.5 minutes)
3. Add 4mL of DMEM + 15% FBS Media to tube
4. Centrifuge at 1500rpm for 5min
5. Resuspend pellet in 8mL of DMEM + 15% FBS media
6. Plate 5mL of sample onto a culture plate
7. Incubate at 37° C and monitor for cell growth
8. If low grow occurs, re-thaw more cells
9. When cells get to confluency of 50-70% passage the culture

Transfection

Perform to transform mammalian cells with DNA vector when cells are at 30-40% confluency
Used Promega FuGENE® HD Transfection Reagent

1. Allow Fugene HD Transfection Reagent to reach room temperature
2. Label a 1.5mL centrifuge tube
3. Recipe per 1mL of cell culture: 1µg of DNA, 3µL of Fugene, 46µL of DMEM media
4. Incubate mixture at room temperature for 15min
5. Add mixture to cell culture
6. Transfer mixture to incubator and monitor expression over a timescale

D. Experiment 1 Protocol

Microscopy

Purpose: to visualize HEK cells to detect growth and fluorescence
Used a VWR VistaVision microscope to visualize our cultures and a Leica DMI3000 B microscope to take pictures and visualize using fluorescence.

1. Remove cell culture from incubator and position over light source
2. Turn on microscope and focus using the knob on the left
3. Confluency is determined either by estimated the % of the brightfield area covered by the cells and their adhering structures or by use of a hemocytometer
4. For the Leica DMI3000 B , pull out the knob on the viewer use the software to visualize.
5. To Look at fluorescent images, turn off the light source and turn on the fluorescence source. Allow 20 min for warm up. Adjust filter to #2.
6. Compare cells seen using the brightfield with the same cells fluorescence

E. Experiment 2 Protocol

Luciferase Assay

Purpose: to quantify the amount of luciferase produced by our system
Used Promega Luciferase Assay System

1. Allow Luciferase Reagent and Passive Lysis Buffer to thaw
2. Remove growth media from cells
3. Rinse culture in 1xPBS. Do not dislodge cells.
4. Add 200µL of Passive Lysis Buffer
5. Tap plate repeatedly and scrape along plate surface to dislodge cells. Transfer lysate to 1.5mL eppendorf tube.
6. Add 3 times 20µL of lysate with 100µL of Luciferase Assay Reagent into a well of black 96-well clear bottom plate.
7. Assess Relative Luminescence using the Tecan Infinite m200 Pro Plate Reader.

Plate Reader

Purpose: To detect and quantify light emission ability of fluorescent and luminescent molecules

Used Tecan Infinite m200 Pro Plate Reader and black 96-well clear bottom plate

1. Power up Tecan Infinite m200 Pro Plate Reader using switch on the back
2. Start up PC and Tecan i-Control software
3. Make sure software detects the device.
4. Insert plate without cover into loading bay of plate reader
5. Make a new program for luminescence level. Enter plate type.
6. Export data and analyze

F. Experiment 3 Protocol

Time Course Set-Up

Purpose: Compare Luciferase and GFP expression of constitutive dual-reporter system

1. Passage HEK293 cells into 2mL of DMEM+10%FBS media in 6 wells of two 6 well plates
2. Allow 1-2 days for cells to reach 30-40% confluency.
3. Transfect (protocol above) 3 wells with promoterless vector, 3 wells with the minimal promoter vector, and 3 wells with the enhanced promoter vector.
4. After 24 hours take pictures of each cell type
    a. Use the Leica DMI3000 B and fluorescence source to determine if cells are fluorescing
    b. Take pictures of control cells and all 3 transfected cultures using brightfield
    c. Take picture of the same field using fluorescent microscope
5. After pictures are taken, lyse cells using 200µL of Passive Lysis buffer
6. Load 96-well plate in triplicate with 20µL if lysate and 100µL of Luciferase Assay Reagent to be analyzed for luciferase and triplicate for 120µL of lysate to be analyzed for GFP
7. Load plate into Tecan Infinite m200 Plate Reader
8. Run a luminescence program on wells to be analyzed for luciferase testing
9. Run a fluorescence program with excitation wavelength of 395nm and emission wavelength of 509nm with steps of 5nm on wells to be analyzed.
10. Repeat steps 4 -9 for 48 hours and 72 hours using the remaining wells on the plates
11. Compare RFU and RLU expression over 72 hours compared to control

G. Experiment 4 Protocol

Lysis Buffer Trials

Purpose: To find a lysis buffer which preserves the fluorescent ability of GFP

1. Grow ten 1mL wells in a 12-well plate
2. Allow 1-2 days for cells to reach 30-40% confluency.
3. Transfect (protocol above) 5 wells with the enhanced promoter vector
4. Allow to grow in incubator for 48 hours
5. Check that transfected cells are fluorescing
6. Apply 400µL of particular lysis buffer to one control well and one transfected well
7. Lysis Buffers:
    a. Tris-Cl with 150mM NaCl, 0.5mM EDTA, .5% Tween 20, pH 7.5
    b. Tris-Cl with 150mM NaCl, 0.5mM EDTA, .5% Tween 20, .5 PMSF, pH 7.5
    c. Passive lysis buffer
    d. 0.2 M HCl
    e. Tris-Cl with 150mM NaCl, 0.5mM EDTA, .5% Tween 20, pH 7.5 (only 200µL)
8. Tap plate repeatedly and scrape along plate surface to dislodge cells. Transfer lysate to 1.5mL eppendorf tube.
9. Plate 3 wells in a black 96-well clear bottom plate with 120µL of each lysate
10. Run a fluorescence program with excitation wavelength of 395nm and emission wavelength of 509nm with steps of 5nm on wells to be analyzed.
11. Compare transfected cells RFU to control cells to determine if fluorescence ability of GFP is preserved after lysing

H. Experiment 5 Protocol

GFP Quantification

Biovision GFP Quantification Kit

1. Dilute 10µL of the Standard to make a 1ng/µL solution. Add 0, 8, 16, 32, and 40µL of each into wells of well plate to generate a standard curve.
2. Lyse the samples using passive lysis buffer
3. Tap plate repeatedly and scrape along plate surface to dislodge cells. Transfer lysate to 1.5mL eppendorf tube.
4. Add 3 times 20µL of lysate with 100µL of GFP Assay Buffer into a well of black 96-well clear bottom plate.
5. Optional: Add 20µL of GFP Quench Solution and incubate at 55° C for 10 min
6. Assess Relative Fluorescence using the Tecan Infinite m200 Pro Plate Reader. With the "fluorescent intensity" setting. Use Excitation frequency of 488nm and emission frequency of 515nm.

Time Course Set-Up

1. Passage HEK293 cells into 1mL of DMEM+10%FBS media in 24 wells of two 12 well plates
2. Allow 1-2 days for cells to reach 30-40% confluency.
3. Transfect (protocol above) 6 wells with promoterless vector, 6 wells with the minimal promoter vector, and 6 wells with the enhanced promoter vector.
4. After 24 hours visualized cells to determine if fluorescing
5. After pictures are taken, lyse one well of each transfected cell type using 200µL of Passive Lysis buffer
6. Use GFP quantification kit (protocol above) on ne well of each transfected cell type
7. Load 96-well plate in triplicate with 20µL if lysate and 100µL of Luciferase Assay Reagent to be analyzed for luciferase and triplicate for 120µL of lysate to be analyzed for GFP
8. Load plate into Tecan Infinite m200 Plate Reader
9. Run a luminescence program on wells to be analyzed for luciferase testing
10. Run a fluorescence program with excitation wavelength of 488nm and emission wavelength of 510nm with steps of 5nm on wells to be analyzed.
11. Repeat steps 4 -9 for 48 hours and 72 hours
12. Compare RFU and RLU expression over 72 hours compared to control

I. Quantification Data

GFP Quantification

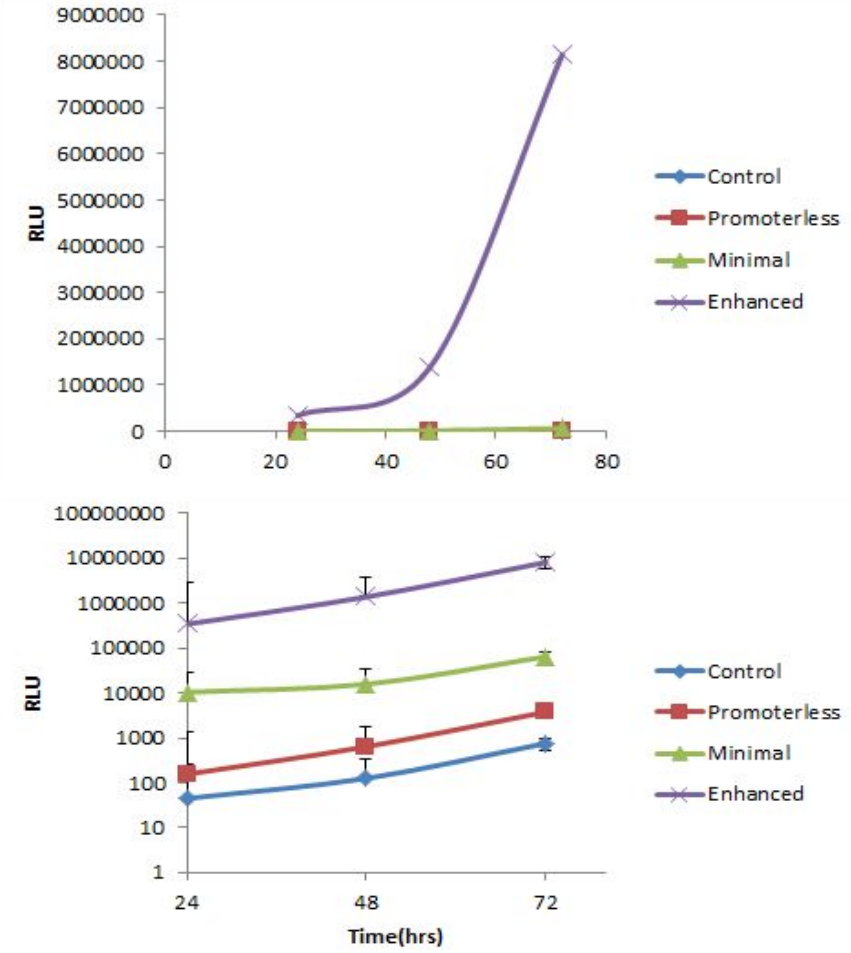
	24 hrs	48 hrs	72 hrs
Control	2,043.33	2,091.33	2,484.67
Promoterless	2,766.67	1,954.67	2,534.00
Minimal	2,722.00	2,737.33	3,111.67
Enhanced	4,324.00	23,719.33	36,445.33

Luciferase Quantification

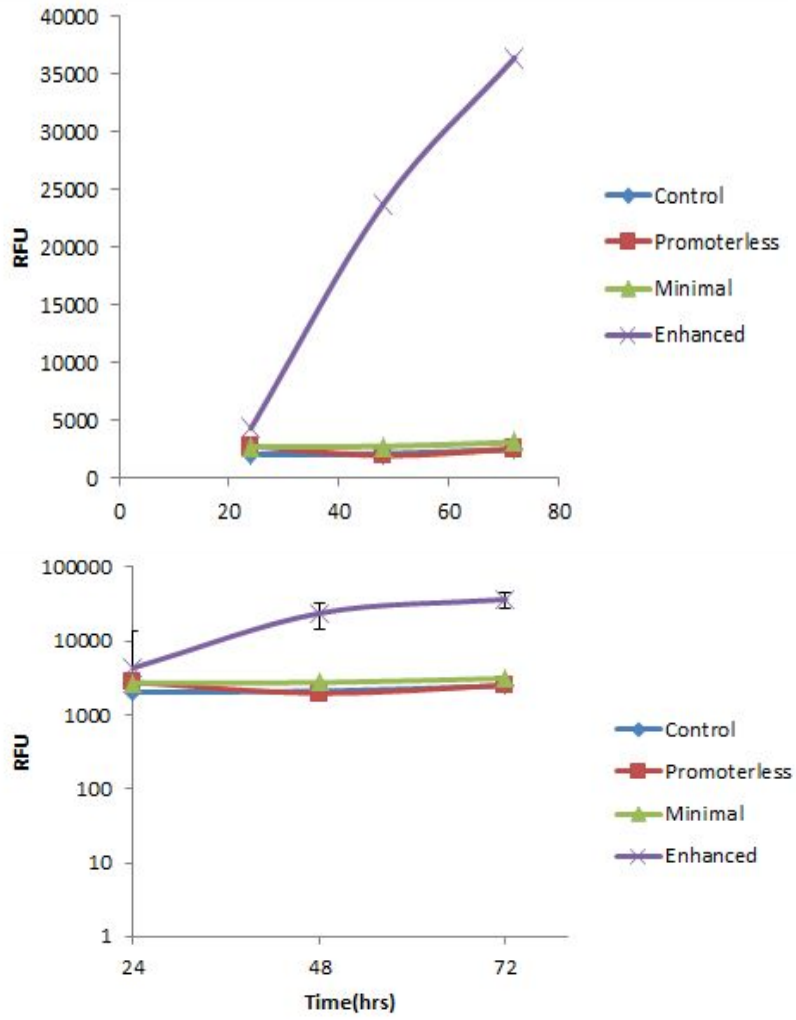
	24 hrs	48 hrs	72 hrs
Control	45.00	126.33	758.33
Promoterless	156.67	640.33	3,811.00
Minimal	10,428.00	15,714.67	64,765.00
Enhanced	342,370.67	1,379,666.67	8,139,761.00

J. Additional Quantification Analysis

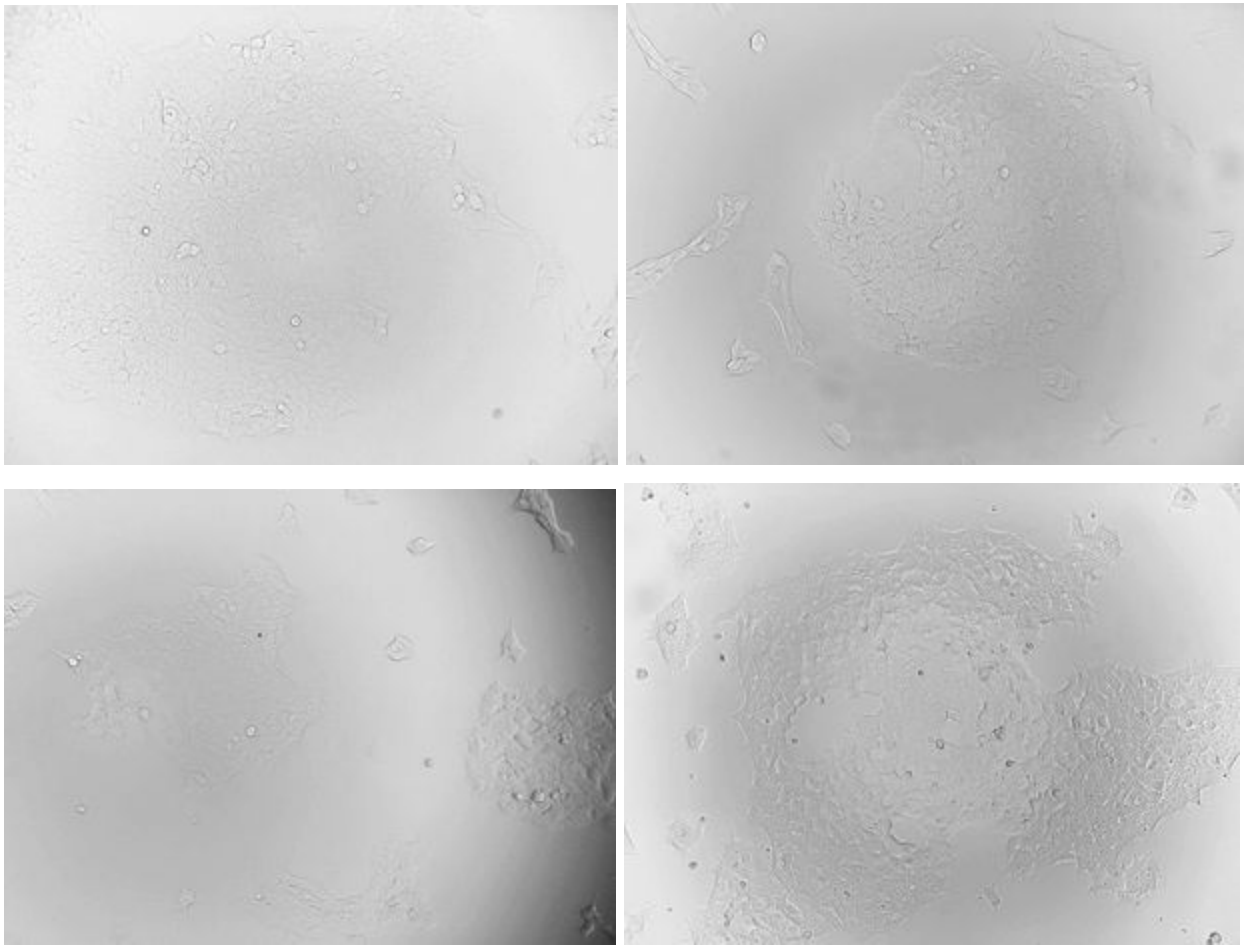
Luciferase Quantification



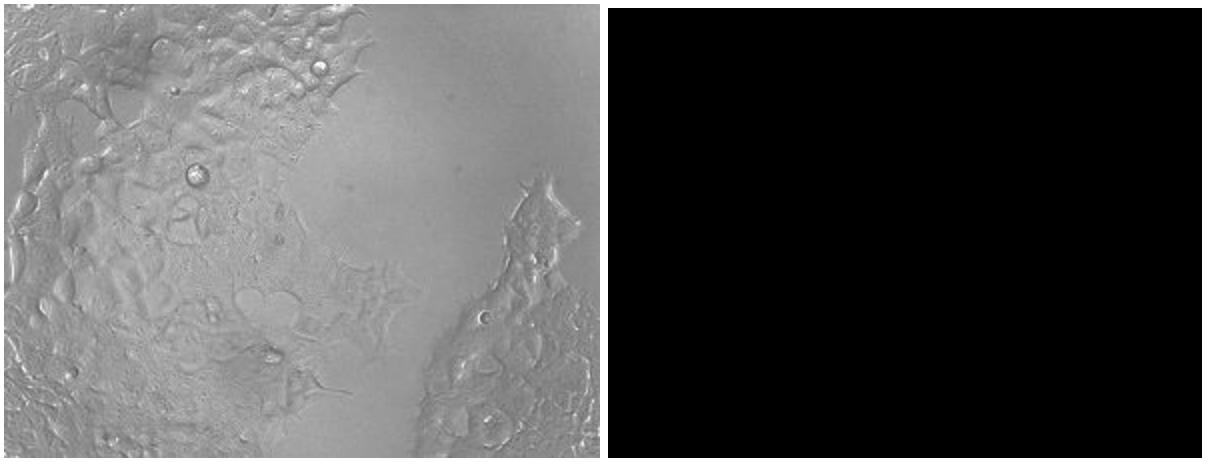
GFP Quantification

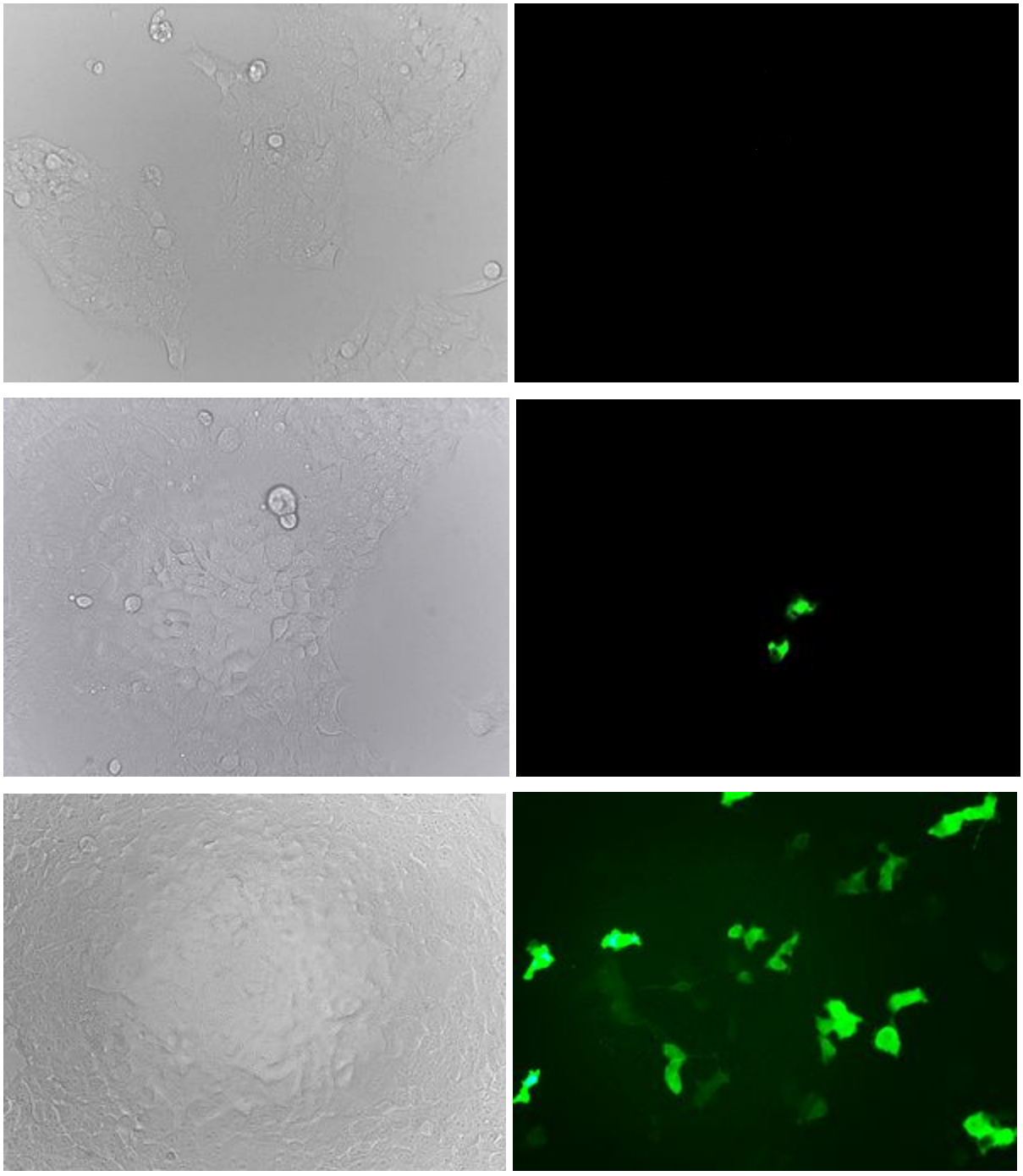


K. GFP Control and Fluorescent Images

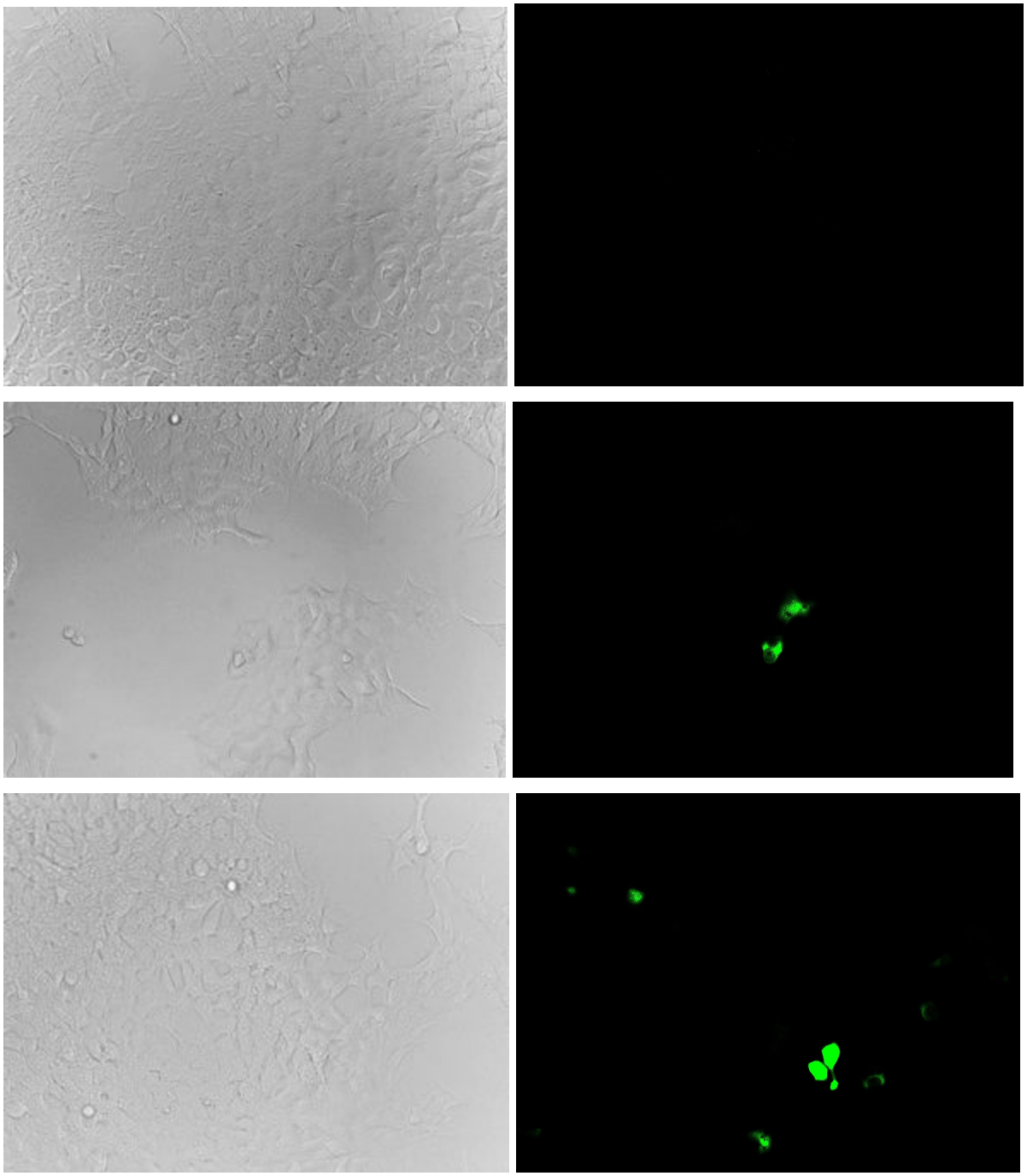


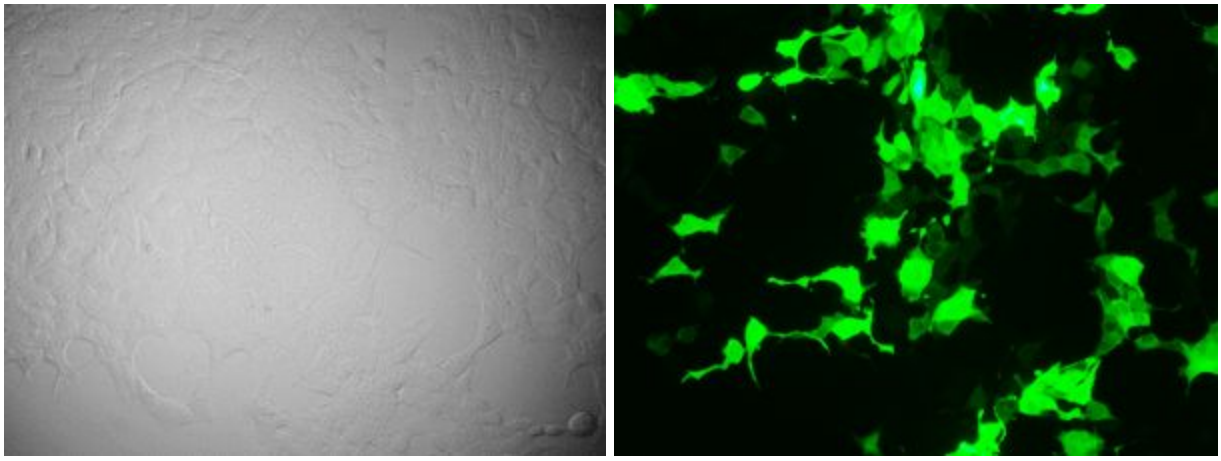
T = 24 hours





T = 48 hours





T = 72 hours